FIBROUS CONNECTIVE TISSUE

Three essential features: (1) cell, (2) fibres, and (3) matrix.

Introduction

Dictionaries do not always give the right definitions of things, especially in relation to the meat trade, and it would be foolish to think that sweetbreads originate from pancreas rather than thymus just

because it says so in some dictionaries (presumably compiled by vegetarians). Even though most dictionaries dictate that gristle is the same thing as cartilage, I disagree. Apart from the odd bit of scapular, joint, or costal cartilage, there is virtually no cartilage in most meat cuts, yet all my life I have found chewy strands of connective **tissue** in tough cooked meat and I have called them "gristle". Whatever name you may give them, I am sure you know what I am writing about, and will agree that we need to understand the scientific basis of the fibrous connective tissues in meat.
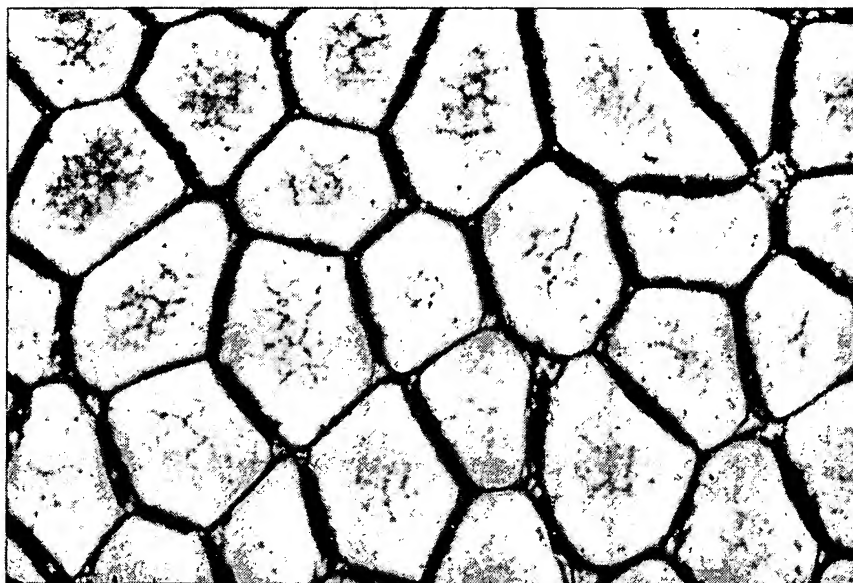
The fibrous connective tissues in meat form a continuous mesh, as shown in the image to the left, from the microscopic strands of endomysium around individual muscle fibers, to the larger layers of perimysium that delineate bundles of muscle fibers, all being gathered and connected to the thick, strong epimysium on the surfaces of individual muscles.

The image below shows a thick layer of perimysium.

The endomysium, perimysium and epimysium contain two types of protein fibers, **collagen** and **elastin**, which now we will consider in detail.

Collagen fibers

Collagen is an elongated protein that forms extremely strong but very small *fibrils* (best seen with an electron microscope). Lots of these **collagen** fibrils are bound together to form **collagen** *fibers* that easily can be seen with a light microscope. When **collagen** fibers form sheets or cables we can see them in the meat without a microscope, and we may detect them as gristle if they do not gelatinize in cooked meat.

Collagen is the most abundant protein in the animal body, and the **collagen** which occurs in meat may

be an important source of meat toughness. Beef carcasses have to be graded by age mainly because of age-related changes in **collagen** that cause meat from older cattle to be tough.

Large amounts of **collagen** are found in animal skin. In pig skin, for example, **collagen** fibers are tightly woven from two directions to form a tightly woven meshwork. **Collagen** is a raw material for major industries in leather, glue and cosmetics.

Under a light microscope, **collagen** fibers in the connective **tissue** framework of meat range in diameter from 1 to 12 micrometres (0.001 millimetre = 1 micrometre). They do not often branch and, when branches are found, they usually diverge at an acute angle. **Collagen** fibers from fresh meat are white, but usually they are stained in histological sections for examination under a microscope. The most common stain for light microscopy is eosin, which stains **collagen** fibers pink. Unstained **collagen** fibers may be seen by polarized light since they are birefringent (light that comes through Polaroid sunglasses is polarized, with all its waves in one plane; birefringent means having two refractive indices). By rotating the plane of polarized light, **collagen** fibers appear bright against an otherwise dark background (when two Polaroid lenses are perpendicular they block most of the light, but **collagen** fibers can rotate the light so that they appear bright). The birefringence of **collagen** fibers in meat is lost at the point during heating when gelatinization occurs. **Collagen** fibers have a wavy or crimped appearance which disappears when they are placed under tension.

Collagen fibers **fluoresce** with a blue-white light when excited with UV light so that the amount of connective **tissue** on a cut meat surface may be measured very rapidly. Peak excitation is around 370 nm so that the prominent 365 nm peak emission of a mercury arc lamp may be used. Some indication of **collagen** fiber diameter may be obtained by spectrofluorometry (measuring the wavelengths of fluorescence) because the fluorescence is quenched (fades) fairly rapidly. Thus, large **collagen** fibers retain a central core with a pre-quenching emission spectrum for longer than small fibers. Fat only fluoresces weakly, to about the same extent as areas of muscle with a low connective **tissue** content. This is how the connective **tissue** content of meat may be measured with a fiber-optic probe for the on-line detection of tough beef. **Collagen** fluorescence increases with animal age so that <u>probe measurements</u> may have a promising future in beef grading, and fiber-optic probe measurements now have been correlated with consumer taste panel evaluation of chewiness in beef . It is important remember, however, that a probe for connective **tissue** cannot account for toughness caused by short sarcomeres or inadequate aging of the meat!

Electron microscopy reveals that **collagen** fibers are composed of parallel bundles of small fibrils with diameters ranging from 20 to 100 nm (0.001 micrometre = 1 nanometre). **Collagen** fibrils typically have diameters which are multiples of 8 nm that may show the manner in which they grow radially. **Collagen** microfibrils (even smaller structures that make up fibrils) may appear to have a tubular structure with an electron-lucent lumen (appearing empty under the electron microscope).

Collagen fibrils are formed from long tropocollagen molecules which are staggered in arrangement but tightly bound laterally by covalent chemical bonds. For electron microscopy, when negatively stained with heavy metals that spread into the spaces between the ends of molecules, **collagen** fibrils appear to be transversely striated. The periodicity of these striations is 67 nm but often shrinks to 64 nm as samples are processed for
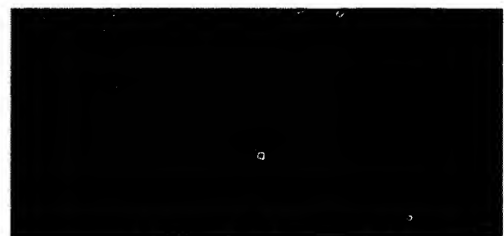
examination. Although **collagen** fibers are located *outside* the
cell, the initial stages of **collagen** fibril assembly may be within the cell, with fibril morphology being regulated by a special site on the fibroblast membrane (cells that form connective **tissue** fibers are called fibroblasts).

Tropocollagen molecules

Tropocollagen is a high molecular weight protein (300,000 Daltons) formed from three polypeptide strands twisted into a triple helix. Each strand is a left-handed helix twisted on itself, but the three strands are twisted into a larger right-handed triple helix. The triple helix is responsible for the stability of the molecule and for the property of self-assembly of molecules into microfibrils. The flexible parts of each strand projecting beyond the triple helix (telopeptides) are responsible for the bonding between adjacent molecules. **In other words, the cross links that bind tropocollagen molecules together laterally are made between the helical shaft of one molecule and the non-helical extension of an adjacent molecule.**

In the polypeptide strands, the small amino acid **glycine** occurs at every third position, and proline and hydroxyproline account for 23% of the total residues. The regular distribution of glycine is required for the packing of tropocollagen molecules and has been claimed as evidence that all animals are derived by evolution from a single ancestral stock, since the chance development of this unique regularity in unrelated animals is thought unlikely. Hydroxyproline is quite rare in other proteins of the body, and an assay for this imino acid (an imino acid is chemically similar, but not the same as an amino acid) provides a measure of the **collagen** or connective **tissue** content in a meat sample. Tropocollagen also contains a fairly high proportion of glutamic acid and alanine as well as some hydroxylysine.

Biochemical types of collagen

Each tropocollagen molecule is composed of three alpha chains but 19 unique alpha chains have been identified, giving rise to 11 different types of **collagen**. These may be categorized into three general classes:

(1) molecules with a long (about 300 nm) uninterrupted helical domain,

(2) molecules with a long (300 nm or greater) interrupted helical domain,

(3) short molecules with either a continuous or an interrupted helical domain.

The various types of **collagen** of interest in understanding the structure of meat are as follows.

- Type I **collagen** forms striated fibers between 80 and 160 nm in diameter in blood vessel walls, tendon, bone, skin and meat. It may be synthesized by fibroblasts, smooth muscle cells (around blood vessels) and osteoblasts (bone-forming cells).

- Type II **collagen** fibers are less than 80 nm in diameter and occur in hyaline cartilage and in intervertebral discs. It is synthesized by chondrocytes (cartilage-forming cells).

- Type III **collagen** forms reticular fibers in tissues with some degree of elasticity, such as spleen, aorta and muscle. It is synthesized by fibroblasts and smooth muscle cells, contributes substantially to the endomysial connective tissues around individual muscle fibers, provides a small fraction of the **collagen** found in skin and occurs in the large **collagen** fibers dominated by Type I **collagen**. It may have some function in regulating **collagen** fiber growth.
- Type IV **collagen** occurs in the basement membranes around many types of cells and may be produced by the cells themselves, rather than by fibroblasts. Although basement membranes were once regarded as amorphous (like glue), many of them now are thought to be composed of a network of irregular cords. The cords contain an axial filament of Type IV **collagen**, ribbons of heparin sulphate proteoglycan, and fluffy material (laminin, entactin and fibronectin). Type IV **collagen** occurs in the endomysium around individual muscle fibers. Instead of being arranged in a staggered array, the molecules are linked at their ends to form a loose diagonal lattice.

- Type V **collagen** is found prenatally in basement membranes and cultures of embryonic cells. It is synthesized by myoblasts (muscle-forming cells), smooth muscle cells and, possibly, by fibroblasts. Type V **collagen** has also been found in the basement membranes of muscle fibers, except at the point where muscle fibers are innervated.

- Type VI **collagen** is a tetramer of Type VI. It forms a filamentous network and has been identified in muscle and skin. The molecule consists of a short triple helix about 105 nm in length with a large globular domain at each end.

Tendons often extend into the belly of a muscle or along its surface before they merge with its connective **tissue** framework, and types I and III **collagen** both may be extracted from meat. Even within tendons, there may be some Type III **collagen** forming the endotendineum or fine sheath around bundles of **collagen** fibrils. In fibers composed of **collagen** Types I and II, fibrils have a straight arrangement whereas, in fibers of Type III **collagen**, the fibrils have a helicoidal arrangement.

Small diameter type III **collagen** fibers are called reticular fibers since, when stained with silver for light microscopy, they often appear as a network or reticulum of fine fibers. The larger diameter **collagen** fibers formed from Type I **collagen** are not blackened by silver.

Collagen fibers shrink when they are placed in hot water, and ultimately they may be converted to gelatin. Around 65oC, the triple helix is disrupted and the alpha chains fall into a random arrangement.

The importance of this change is that it tenderizes meat with a high connective **tissue** content. Tropocollagen molecules from older animals are more resistant to heat disruption than those from younger animals. In early studies, it was suggested that reticular fibers, unlike **collagen** fibers, did not yield gelatin when treated with moist heat. The original suggestion that reticular fibers survive unchanged after cooking is wrong, but a modification of the idea is plausible. Since a piece of meat may contain different types of **collagen**, and since these types may differ in the thermal stability of their cross links, it is possible that, at an intermediate level of cooking around 65oC, endomysial **collagen** and perimysial **collagen** may differ in the extent to which they are affected by the cooking treatment. Heat-induced solubilization of Type I **collagen** is more important in improving meat tenderness by cooking than is the effect of heat on Type III **collagen**.

Collagen biosynthesis

The synthesis of the different polypeptide strands that are combined to make different types of **collagen** is genetically regulated by the production of messenger RNA. The synthesis of polypeptide strands occurs on membrane-bounded polysomes, but the hydroxylation of lysine and proline occurs after the strands are assembled. Ascorbic acid is required for the hydroxylation of lysine and proline. Polypeptide strands enter the cisternae of the endoplasmic reticulum (a membraneous assembly labyrinth within the cell), the terminal extensions of the strands are aligned, and then the strands spiral around each other. Procollagen or immature **collagen** has long terminal extensions protruding from each end of the newly formed triple helix. Procollagen moves to the golgi apparatus and is packaged into vesicles that are moved to the cell surface, probably by microtubules. Except for some Type III procollagen molecules, the long terminal extensions are then enzymatically reduced in length.

Outside the cell, **collagen** molecules become aligned in parallel formations, and then they link up laterally to form fibrils. It is likely that tropocollagen monomers are partially assembled together in groups before they are added to an existing **collagen** fibril. Firstly, vacuoles containing procollagen fuse to form a fibril-containing compartment. Then the cytoplasmic extensions withdraw from between several fibril-forming compartments to create a bundle-forming compartment. Sometimes **collagen** fibrils occur intracellularly, but it is not clear whether this is **collagen** taken up by phagocytosis (engulfed by the cell) or a surplus of newly synthesized **collagen**.

The characteristic parallel staggered arrangement of tropocollagen molecules in a **collagen** fibril is caused by the 67 nm repeating pattern of oppositely charged amino acids along the length of the tropocollagen molecule. The degree of overlapping of adjacent molecules and the gaps left between the ends of molecules cause the striated appearance of **collagen** fibers seen by electron microscopy. The fibroblasts of young animals are metabolically more active than those of older animals, particularly for aerobic metabolism.

Accumulation of collagen in meat

Although the relative proportions of Types I and III collagen in a muscle may be related to meat tenderness, the overall amount of **collagen** and its degree of crosslinking also are important. The absolute amount of **collagen** in an animal may increase as animals become older, and this may have an effect on meat toughness, but rapid growth of muscle fibers also may dilute the relative amounts of **collagen** in meat. Considering the supposed importance of **collagen** in meat toughness, the absence of

overwhelming evidence from taste panel studies is rather curious. It seems reasonable that stewing beef is tougher than prime steak because it has more **collagen**, but is **collagen** responsible for differences in tenderness between the same cut of steak from different carcasses? Recent studies with UV fiber-optics suggest that it is, because this new technology allows us to see overall trends that are difficult to identify by chemical analysis of small samples of meat.

Within a carcass, there may be considerable differences in **collagen** content between different muscles and this is reflected in their retail price. **Collagen** content also may differ between sexes. For example, the hydroxyproline content is higher in pork from females than castrated males. However, the amount of **collagen** in meat, when expressed as a proportion of wet sample weight, also is affected by fat content. In steaks from a veal carcass, for example, the **collagen** content might exceed 0.5%, but could be much less in the same region from a steer carcass in which fat had accumulated to "dilute" the **collagen** content.

Collagen in meat may be studied by measuring **collagen** fibril diameters in electron micrographs. In tendons, fibril diameters in the fetus are unimodal but become bimodal in the adult. Large diameter fibrils may have more intrafibrillar covalent cross-links, while small diameter fibrils may have more interfibrillar non-covalent cross links. Thus, fibril diameter may be related to fibril strength and elasticity. Meat with large diameter **collagen** fibers tends to be tougher than meat with thinner **collagen** fibers.

Little is known about the mechanisms by which **collagen** fibers become arranged in a muscle, or about the interactions which occur between fibroblasts and the fibers that they produce, although it is possible that glycosaminoglycans play some part in this interaction.

Collagen is very important in muscle development. Myoblasts, the cells that form muscle fibers, develop a parallel alignment when cultured on a substrate of Type I **collagen**, but they do not become elongated or aligned on Type V basement membrane **collagen**. Myoblasts may themselves form Types I, III and V **collagen**, while myotubes (immature muscle fibers) also may form **collagen**, but only when associated with fibroblasts. The identification of **collagen** in developing muscle is complicated by the fact that the tail unit of the acetylcholinesterase molecule (involved in neural control of muscle contraction) has a **collagen**-like sequence that contains hydroxyproline and hydroxylysine.

Crosslinking of collagen molecules

Within an individual **collagen** molecule, the three polypeptide strands are linked together by stable intramolecular bonds that originate in the non-helical ends of the molecule.

The great strength of collagen fibers, however, originates mainly from the stable intermolecular covalent bonds between adjacent tropocollagen molecules.

Stable disulphide bonds between cystine molecules in the triple helix also occur. During the growth and development of meat animals, covalent cross links increase in number, and **collagen** fibers become progressively stronger. Meat from older animals, therefore, tends to be tougher than meat from the same

region of carcasses from younger animals. This relationship is complicated in young animals by the rapid synthesis of large amounts of new **collagen**. New **collagen** has fewer cross links so that, if there is a high proportion of new **collagen**, the mean degree of cross linking may be low, even though all existing molecules are developing new cross links. As the formation of new **collagen** slows down, the mean degree of cross linking increases. Another complication is that many of the intermolecular cross links in young animals are reducible (the **collagen** is strong but is fairly soluble). In older animals, reducible cross links are probably converted to non-reducible cross links (the **collagen** is strong but is far less soluble and more resistant to moist heat). The chemistry of these changes is still a subject for debate.

Pyridinoline, a non-reducible cross-link, may be involved in the increased heat stability of epimysial connective tissues from older animals. Although changes in **collagen** solubility might be an important factor affecting the tenderness of beef from older animals, the effect in younger animals at a typical commercial slaughter weight may be relatively slight. However, relative to increasing maturity levels used in US beef grading, the pyridinoline content and thermal stability of intramuscular **collagen** both increase.

Differences in the degree of cross linking may occur between different muscles of the same carcass, and between the same muscle in different species. For example, **collagen** from the longissimus dorsi is less cross-linked than **collagen** from the semimembranosus, and **collagen** from the longissimus dorsi of a pork carcass is less cross-linked than **collagen** from the bovine longissimus dorsi. Nutritional factors such as high-carbohydrate diet, fructose instead of glucose in the diet, low protein, and pre-slaughter feed restriction may reduce the proportion of stable cross links. Nonenzymic glycosylation (a reaction between lysine and reducing sugars) may be involved in the interaction between diet and **collagen** strength. In general, the turnover rate of **collagen** is accelerated in cattle fed a high energy diet. The rate of **collagen** turnover in skeletal muscle may be about 10% per day and the turnover time for **collagen** may be inversely proportional to **collagen** fibril diameter.

Elastin and elastic fibers

Individual **collagen** fibers only lengthen by about 5% when stretched and little elasticity is possible where **collagen** is formed into cable-like tendons. However, much of the **collagen** that is present in meat forms a meshwork so that stretching of the whole meshwork is possible because its configuration changes. Fibers with truly elastic properties, however, are necessary in structures such as the ligamentum nuchae of the neck and the abdominal wall. And all arteries, from the aorta down to the finest microscopic arterioles, rely on **elastin** fibers to accommodate the surge of blood from contraction of the heart. **Elastin** fibers may be stretched to several times their original length but rapidly resume their original length once released. **Elastin** is found in all vertebrates except primitive jawless fish, and in evolution it appeared first in cartilaginous fish. The **elastin** fibers in the image below (from loose connective **tissue** around the intestine) are the thin black ones. The much thicker, red-brown fibres are **collagen**.
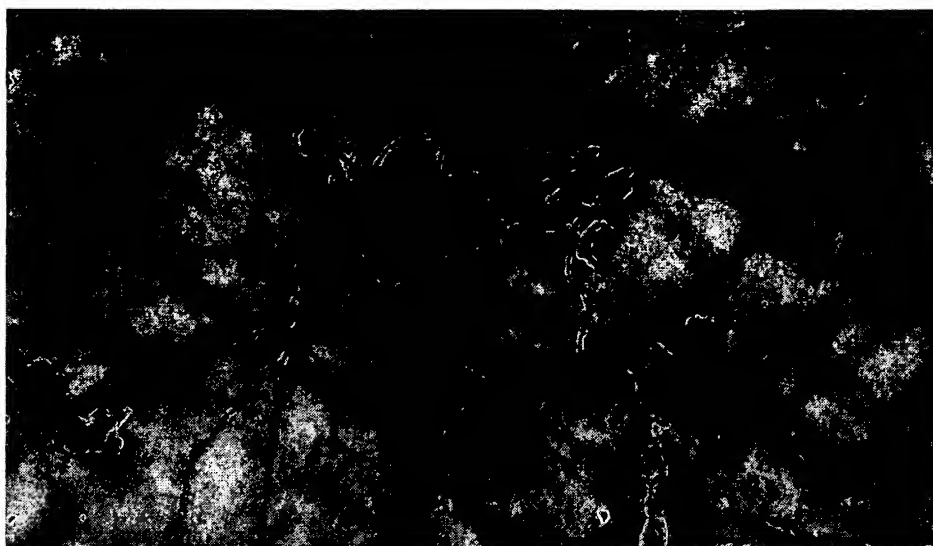
Elastic fibers are made of the protein **elastin**.

Elastin resists severe chemical conditions, such as the extremes of alkalinity, acidity and heat that destroy collagen.

Fortunately, there are relatively few elastic fibers in muscle, otherwise cooking would do little to reduce meat toughness. The **elastin** fibers in muscles that are used frequently for locomotion are larger and more numerous than those of less frequently used muscles. **Elastin** fibers in the epimysium and perimysium of beef muscles range from 1 to 10 microns in diameter. **Elastin** is synthesized by arterial smooth muscle cells, but the origin of **elastin** in non-vascular locations is not properly understood. In the lung, for example, large amounts of **elastin** are synthesized by various types of lung cells but the cellular source of the **elastin** fibers in meat is unclear at present. Some elastic fibers in muscle are involved in the attachment of sensory organs called neuromuscular spindles.

Elastic fibers usually are pale yellow. When elastic fibers are stretched, they may become visible in polarized light without staining, but this requires careful attention to the refractive index of the mounting medium. In the bovine ligamentum nuchae, the pattern of birefringence indicates that there are two micellar structures, one arranged circularly on the outside and the other arranged axially in the centers of the fibers. Elastic fibers in meat have a small diameter (approximately 0.2 to 5 microns) although they are much larger in the ligamentum nuchae. Elastic fibers in the connective **tissue** framework of meat are usually branched.

Electron microscopy reveals that elastic fibers are composed of bundles of small fibrils approximately 11 nm in diameter embedded in an amorphous material. In the bovine ligamentum nuchae, fibrils may be constructed from smaller units or filaments approximately 2.5 nm in diameter. **Elastin** filaments are bound by non-covalent interactions to form a three-dimensional network and elastic fibers are assembled in grooves on the fibroblast surface where initially rope-like aggregations of fibrils become infiltrated with amorphous **elastin**. Unlike the situation in elastic ligaments, where **elastin** forms fibers, the **elastin** of the arterial system occurs in sheets that condense extracellularly in the absence of fibrils.

Although **elastin** resembles tropocollagen in having a large amount of glycine, it is distinguished by the presence of two unusual amino acids, desmosine and isodesmosine. Like **collagen, elastin** contains hydroxyproline, although it may not have the same function of stabilizing the molecule. Tropoelastin, the soluble precursor molecule of **elastin** (molecular weight 70,000 to 75,000), is secreted by fibroblasts after it has been synthesized by ribosomes of the rough endoplasmic reticulum and processed by the Golgi apparatus. In the presence of copper, lysyl oxidase links together four lysine molecules to form a desmosine molecule. Isodesmosine is the isomer of desmosine. The aorta may be fatally weakened by a lack of mature **elastin** in animals deprived of dietary copper. **Elastin** in the arterial system is produced by smooth muscle cells instead of fibroblasts.

The functional properties of **elastin** in different tissues such as lung and aorta may be related to differences in the ratio of tropoelastin A to B. The **elastin** of elastic cartilage might be a different genetic type to that found in the vascular system but, overall, the diversity of different genetic types of **elastin** is far less than for **collagen**.

The cells of fibrous connective tissue

The dominant cell type in the fibrous connective **tissue** of meat is the fibroblast, but other cells also exist. Macrophages or histiocytes are sometimes quite numerous and, when inactive, may resemble fibroblasts in appearance. However, the motility of macrophages is soon revealed by **tissue** inflammation or the injection of colloidal dyes. Macrophages migrate through the **tissue** and act as scavengers by engulfing invasive microorganisms or foreign particles by phagocytosis.

Cells from the vascular system may wander through connective tissues and even compact structures such as tendons have their own lymphatic and vascular supply, something that is not easily seen in an exsanguinated carcass. The vascular cells include a variety of lymphocytes and the plasma cells responsible for antibody production. Eosinophils are cells with bilobed nuclei and numerous cytoplasmic granules readily stained by eosin. The skeletal muscles of cattle, and sometimes sheep, may become inundated with eosinophils (eosinophilic myositis). The affected areas appear as irregular pale lesions and often are detected by meat inspectors looking for muscle parasites. Eosinophils may be attracted to areas of antibody activity and eosinophilic myositis may be an allergic response.

Located around the body are some very interesting cells called mast cells. They are involved in a variety of vital body functions, like resisting disease, but they might also have a special importance for the meat industry. Mast cells occur within the skeletal muscles of meat animals, mainly in the perimysium and epimysium. The numbers of mast cells may be increased in pathological situations and, in denervated muscle, mast cells may move from the central tendon into the belly of the muscle. The cytoplasm of mast cells contains large numbers of metachromatic granules (metachromasia is a color change of dyes such as methylene blue so that metachromatic granules are purple while the surrounding **tissue** is blue). Mast cells contain heparin and histamine. Heparin prevents the coagulation of blood and histamine increases the permeability of small blood vessels. Heparin also activates the enzyme lipoprotein lipase involved in the accumulation of triglyceride by adipose cells, so there could be some relationship between the distribution of mast cells and the availability of fatty acids for storage in marbling fat in meat. Mast cells also may release a substance that activates cell division in nearby cells. Thus, in both availability of fatty acids for storage and in the formation of new fat cells, the development of intramuscular marbling fat in meat may have some relationship to the distribution of mast cells. Mast cells sometimes come into close contact with skeletal muscle fibers, but most mast cells are located along fine branches of the lymphatic system in the perimysium and endomysium. Mast cells also have

been implicated in the regulation of collagenase activity and, thus, may have part to play in the turnover of **collagen** and its cooking-resistant strength.

Why meat must be a natural food for us

My favourite gem of information about connective **tissue** concerns the digestibility of **elastin**. During the digestion of meat in the human gut, elastic fibers are broken down by elastase, an enzyme from the pancreas that would not be there if our evolutionary ancestors had not been at least partly carnivorous. In other words, I have never read of the occurrence of **elastin** in any human food except meat. So if we have evolved a highly specific enzyme, elastase, to deal with **elastin** in our food, this can only mean that we are the descendants of meat eaters.

The meat industry always seems to be under attack from the popular press with a stream of bad news stories questioning meat in the human diet. Thus, I derive great peace of mind from knowing scientifically that meat must be a natural component of my diet. This fits nicely with my intuitive belief that, thousands of years ago, my ancestors worked hard all day running down something tasty to bring back to the family, and that the best part of the day was sitting around a camp fire gnawing on a chunk of partly burnt meat, chewing the fat, and washing it down with home-brew.

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